

Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions

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The purpose of this study was to determine whether oligonucleotides the size of siRNA are permeable to gap junctions and whether a specific siRNA for DNA polymerase β (pol β) can move from one cell to another via gap junctions, thus allowing one cell to inhibit gene expression in another cell directly. To test this hypothesis, fluorescently labelled oligonucleotides (morpholinos) 12, 16 and 24 nucleotides in length were synthesized and introduced into one cell of a pair using a patch pipette. These probes moved from cell to cell through gap junctions composed of connexin 43 (Cx43). Moreover, the rate of transfer declined with increasing length of the oligonucleotide. To test whether siRNA for pol β was permeable to gap junctions we used three cell lines: (1) NRK cells that endogenously express Cx43; (2) M β 16tsA cells, which express Cx32 and Cx26 but not Cx43; and (3) connexin-deficient N2A cells. NRK and M β 16tsA cells were each divided into two groups, one of which was stably transfected to express a small hairpin RNA (shRNA), which gives rise to siRNA that targets pol β . These two pol β knockdown cell lines (NRK-kcdc and M β 16tsA-kcdc) were co-cultured with labelled wild type, NRK-wt or M β 16tsA-wt cells or N2A cells. The levels of pol β mRNA and protein were determined by semiquantitative RT-PCR and immunoblotting. Co-culture of M β 16tsA-kcdc cells with M β 16tsA-wt, N2A or NRK-wt cells had no effect on pol β levels in these cells. Similarly, co-culture of NRK-kcdc with N2A cells had no effect on pol β levels in the N2A cells. In contrast, co-culture of NRK-kcdc with NRK-wt cells resulted in a significant reduction in pol β in the wt cells. The inability of M β 16tsA-kcdc cells to transfer siRNA is consistent with the fact that oligonucleotides of the 12 nucleotide length were not permeable to Cx32/Cx26 channels. This suggested that Cx43 but not Cx32/Cx26 channels allowed the cell-to-cell movement of the siRNA. These results support the novel hypothesis that non-hybridized and possible hybridized forms of siRNA can move between mammalian cells through connexin-specific gap junctions.

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Gap junction channels permit the propagation of action potentials from cell to cell in the heart (Barr *et al.* 1965), and provide the structural and functional basis for electrical synaptic transmission between nerve cells (Bennett, 1977). In addition to facilitating current flow from cell to cell, gap junction channels composed of connexins allow the passage of larger solutes, including second messengers (Tsien & Weingart, 1976; Loewenstein, 1981; Elfgang *et al.* 1995; Goldberg *et al.* 1999; Valiunas *et al.* 2002), polypeptides (Simpson *et al.* 1977) and nucleotides, such as ATP, cAMP and ADP (Pitts, 1998; Bevans & Harris, 1999;

Goldberg *et al.* 1999, 2004). The transfer of larger solutes, such as RNA, was reported by Kolodny (1971), but this macromolecular transfer is now generally believed to arise from the transfer of degradation products or via exocytosis and endocytosis. Until now it has not been known whether larger molecules, such as oligonucleotides of similar size to short interfering RNA (siRNA), can pass from cell to cell via gap junctions.

Endogenous and exogenous siRNAs, 20–30 nucleotides in length, can profoundly affect gene expression (Elbashir *et al.* 2001; Caplen & Mousses, 2003; Miller & Grollman, 2003; Xu *et al.* 2004). Hence siRNA is important in regulating cellular function and has potential therapeutic applications (Hampton, 2004). In plants, siRNA can

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travel from cell to cell (Yoo *et al.* 2004), resulting in system-wide silencing of specific genes. The inter-cellular path in plants that is exclusive of the extracellular space is the plasmodesmata with a pore diameter of 100–200 nm, a size more than adequate for the passage of siRNA. It is not found in either invertebrates or vertebrates.

The analogous structure in animals is the gap junction. In invertebrates, the subunit proteins of gap junction channels are the innexins, while in vertebrates they are the connexins and pannexins (White *et al.* 2004; Bruzzone *et al.* 2005). Gap junction channels have pore diameters between 1.0 and 1.5 nm. This is close to the minor diameter (width) of siRNA, so it is possible that siRNA could permeate gap junction channels, but there has been no direct demonstration of this in vertebrates or invertebrates. For example, in the invertebrate *C. elegans*, ingestion of siRNA resulted in systemic effects (Kittle & Buchholz, 2003), but the mechanism by which the siRNA was delivered to cells not lining the alimentary canal has not been defined and does not appear to involve the transmembrane protein SID-1 that mediated transport of RNA interfering (RNAi) (Feinberg & Hunter, 2003).

Endogenous and exogenous siRNAs survive and remain functional for hours or days (Alisky & Davidson, 2004). Given their prolonged survival, if siRNAs permeate gap junction channels then they would influence not only the cell in which they were produced, but also adjacent and perhaps even distant cells of a syncytium. Thus a small group of cells could potentially use this mechanism to alter organ function. An extension of this idea is that a small group of cells implanted in a tumour could use this mechanism to inhibit tumour growth. Recent reports have demonstrated successful targeting of genetically engineered mesenchymal stem cells to tumours where they produced interferon (Studney *et al.* 2004). Thus, the ability to deliver siRNA to the interior of a cell in a target tissue exclusive of the extracellular space has significant therapeutic potential. The purpose of the present study was to determine whether siRNA can permeate gap junctions and thus allow one cell to regulate gene expression in another cell.

Methods

Dual patch clamp

We used dual whole cell patch clamp to assess junctional conductance (Neyton & Trautmann, 1985). To determine the permeability of the oligonucleotides we employed the methods of Valiunas *et al.* (2002). Cells were bathed in a solution containing 150 mM NaCl, 10 mM KCl, 2 mM CaCl₂, 5 mM Hepes (pH 7.4) and 5 mM glucose. Pipettes contained 120 mM potassium aspartate, 10 mM NaCl, 3 mM MgATP, 5 mM Hepes (pH 7.2) and 10 mM EGTA.

Oligonucleotides were added to the pipette solution to concentrations of 0.25–0.5 mM. We calibrated the camera system for the morpholinos as we have previously reported for Lucifer Yellow (Valiunas *et al.* 2002). The effective range was 300 nm to 0.5 mm for the cell types used.

Cell lines and culture conditions

The mouse embryonic fibroblast line M β 16tsA (Gu *et al.* 1994), rat kidney line (NRK), mouse neuroblastoma line (N2A) and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, at 34°C (M β 16tsA) or 37°C (NRK, HeLa and N2A) in a 5% CO₂ humidified air atmosphere. Human mesenchymal stem cells were purchased from Clonetics/BioWhittaker (Walkersville, MA, USA) and cultured as previously described (Potapova *et al.* 2004).

The vector, pH1P-pgkneoB (Polosina *et al.* 2004), directs expression of a short hairpin RNA (shRNA) that has been shown to decrease the level of DNA polymerase β (pol β) RNA greater than 90% (less than 10% pol β remaining). pH1P-pgkneoB (1 μ g) was transfected into NRK cells using FUGENE according to the manufacturer's protocol (Roche) and clones were selected with geneticin (G418). Individual clones were isolated and the levels of pol β mRNA and protein were determined by semiquantitative RT-PCR and immunoblotting. The clone with the lowest level of pol β (NRK-kcdc) was selected for the experiments described. Pol β knockdown M β 16tsA cells (M β 16tsA-kcdc) were made as described by Polosina *et al.* (2004). The siRNA for pol β is a 22-mer with a length of slightly more than 7.0 nm.

Western blot analysis

Cell lysates were prepared from confluent monolayer cells as described by Biade *et al.* (1998). Equal amounts of cellular protein (70 or 50 μ g) were resolved by 10 or 12% SDS-PAGE and transferred to nitrocellulose or Immobilon-PVD (Bio-Rad) membranes. Blots were incubated with one of the following antibodies: 18S monoclonal anti-pol β (NeoMarkers, Fremont, CA, USA, 669P201), anti-actin (Santa Cruz, Santa Cruz, CA, USA, sc-7210), anti-Cx43, anti-Cx26 and anti-Cx32 (Zymed Laboratories Inc., San Francisco, CA, USA, 71-0700, 71-0500 and 71-0600, respectively). Immunoblots were carried out with secondary antibody conjugated to horseradish peroxidase (Santa Cruz), detected by a MB chemiluminescence kit (Pierce, Rockford, IL, USA).

RT-PCR analysis

RNA was isolated from cells using RNeasy MinElute (Qiagen, Valencia, CA, USA, catalogue no. 74204).

RT-PCR was performed using SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA, USA, catalogue no. 10928-042) with 1 μ g of RNA as template and products subjected to electrophoresis in a 1% agarose gel. Primers for pol β were (5'-GACATGCTCAC-AGAACTCG-3', 5'-CGGATGGTGTACTCATTGAT5-3'), and for actin they were (5'-ACAGATCATGTTTGAGACC-3', 5'-CCACCGATCCACACAGAGTA-3').

Flow cytometry

To determine how pol β mRNA levels of wild type (wt) were altered due to contact with siRNA-expressing cells (kcdc), wt and kcdc cells were grown together for several days and then separated by fluorescence activated cell sorting (FACS). Before cells were mixed, either the wt or kcdc cells were stained using the Vybrant CFDA SE Cell Tracer Kit according to the manufacturer's protocol (Molecular Probes). Wt and kcdc cells were mixed in proportion 1:1 or 1:2 for NRK, 1:6 for M β 16tsA, 1:2 for N2A/M β 16tsA-kcdc and NRK/M β 16tsA-kcdc, and 1:1 for NRK-kcdc/N2A. The mixed cultures were grown together for 6–8 days. Cells were trypsinized and resuspended in cold PBS containing 2 mM EDTA. Separation of unlabelled, non-fluorescent cells from positive, highly fluorescent cells was performed on a Becton Dickinson FACS Vantage model flow cytometer equipped with an air-cooled argon laser emitting 488 nm laser light. Fluorescence signals were collected using a 530 nm wavelength filter with a 30 nm bandpass. Cells with low fluorescence intensities were discarded to ensure that both the non-fluorescent and highly fluorescent cells were not significantly contaminated with the other cell type. RNA was isolated from the sorted cells and control cells that had been grown separately and analysed by RT-PCR.

Morpholino modelling

A tri-guanine base morpholino model was generated using the Dundee ProDrg server (Schuettelkopf & van Aalten, 2004). The model was energy minimized using the Gromacs (v3.2: Berendsen *et al.* 1995; Lindahl *et al.* 2001; van der Spoel *et al.* 2004) molecular simulation package with molecular topology files generated by the ProDrg server. The starting ProDrg structure was first solvated with water molecules using the program GENBOX. Following a 1 ps (picosecond) minimization run using the MDRUN program and the Gromacs force field, ffGMX, the solvated system was equilibrated with a 20 ps minimization run in which positional restraints were placed on the morpholino. The solvated morpholino was then subjected to a 50 ps simulated annealing run at room temperature. For comparison, the coordinates of DNA and RNA were obtained from the Protein Data Bank (PDB)

and Nucleic acid Database (NDB), respectively, at Rutgers University. All structures were visualized with PYMOL (pymol.sourceforge.net).

Results

Cell-to-cell transfer of synthetic oligonucleotides

To test the hypothesis that rod-shaped molecules of the size range of siRNA are permeable to gap junction channels, we used oligonucleotides (constructed by Morpholino Inc.) of three lengths, 12-, 16- and 24-mer. Their sequences had subunits consisting of a nucleic acid base, a morpholine ring and a phosphoramidate linker. All three lengths were constructed to ensure that they would not readily hybridize or be degraded by cellular nucleases (Mudziak *et al.* 1996; Summerton & Weller, 1997). Each probe was labelled with carboxyfluorescein on its 3' end and was subjected to HPLC to separate any unattached carboxyfluorescein from the labelled oligonucleotide. Based on previous research (e.g. Summerton & Weller, 1997) it is unlikely that degradation of the morpholino and the removal of the tag occurred in the cytoplasm. We also ran HPLC on our nucleotides in the pipette before and after using them in experiments and could find no free tag.

The three sequences used in our study are: 12-mer, 5'-CCTCTTACCTCA-3'; 16-mer, 5'-CCTCTTACCTCAGTTA-3'; and 24-mer, 5'-CCTCTTACCTCACCTTACCTCA-3'. All three oligonucleotides had minor diameters of ~ 1.0 nm and length ranging from 3.8 (12-mer) to 7.6 nm (24-mer). The minor diameter and approximate lengths were determined as described in the Methods. The minimized morpholino model has a single stranded width of 10.7 Å and base stacking distance of 4 Å. In comparison, the width of a single stand of B-DNA (PDB identification no. 1DPN) is 11 Å and that of single-stranded RNA (NDB identification no. 472d) is 10 Å. The base stacking distances of RNA and DNA are 4 and 3 Å, respectively.

In cells, siRNA can exist in either single-stranded or double-stranded forms, which will comprise an equilibrium distribution. Since the single-stranded siRNA has the smaller minor diameter, it would be expected to permeate gap junction channels more easily than the double-stranded form. To test this, a compliment to the 12-mer was synthesized and combined with our original 12-mer, resulting in the formation of a double-stranded oligonucleotide whose permeability could be tested in the same fashion as that of the single-stranded form. We used HeLa cells expressing Cx43 (Valiunas *et al.* 2004), M β 16tsA-kcdc and M β 16tsA cells expressing Cx26/Cx32, or human mesenchymal stem cells expressing Cx43 (Valiunas *et al.* 2004) to probe for oligonucleotide transfer of the 12-, 16- and 24-mers.

Figure 1A shows an example of oligonucleotide transfer in HeLa cells. The transfer is visualized from source cell to recipient cell over a 12 min interval for the 12-mer. The inset in the right-hand panel shows the junctional current measured in response to a voltage step for the same cell pair. The measured conductance was 40 nS. Figure 1B shows a lack of transfer for a M β 16tsA-kcdc cell pair after a 20 min interval. The junctional conductance for this pair was 30 nS. These cells express Cx32 and Cx26 but not Cx43 (see Fig. 3). For both M β 16tsA-kcdc ($n = 5$) and M β 16tsA (wt) cells ($n = 5$) no transfer of 12-mer was observed. In four cases (2 M β 16tsA-kcdc and

2 M β 16tsA (wt) cells) where junctional conductance was greater than 25 nS (range 25–40 nS), no transfer of 12-mer was observed over 20–30 min intervals. The data shown in Fig. 1B are from one of these experiments. Oligonucleotide transfer was also observed between human mesenchymal stem cells (Fig. 1C) where the junctional conductance was 17 nS. Figure 1D illustrates the transfer of the 24-mer 40 min after injection. The junctional conductance was 35 nS. In Fig. 1E the source cell has been removed, leaving the recipient cell. HeLa and hMSCs cells have been shown to express Cx43 (Valiunas *et al.* 2004). These data suggest that synthetic nucleotides selectively pass through Cx43

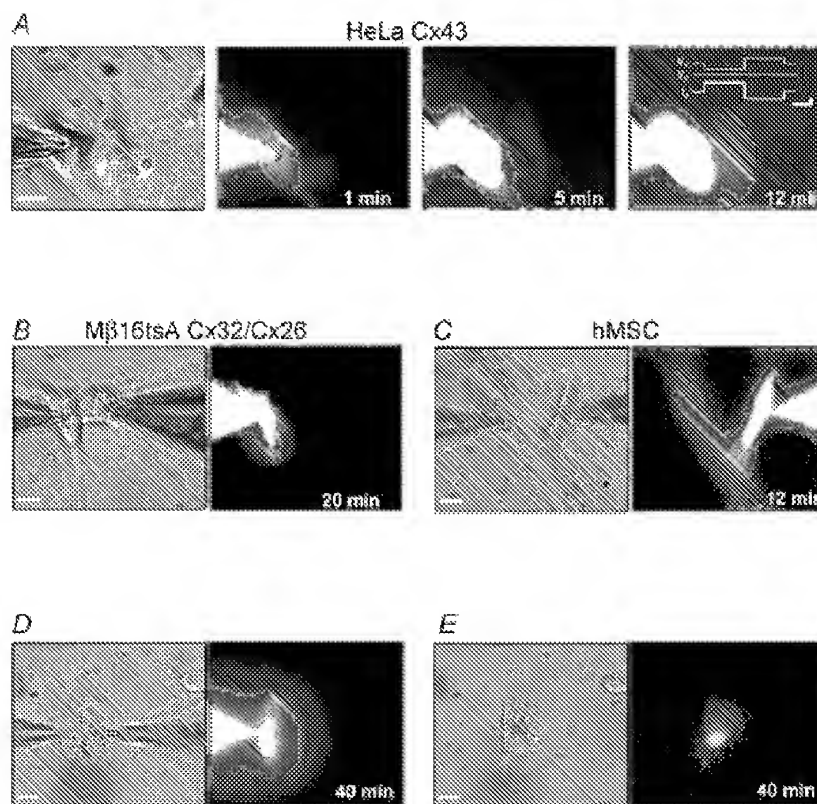


Figure 1. Oligonucleotide transfer through gap junctions

A, HeLa cell pair transfected with Cx43 is used for this illustration. Fluorescent images of a cell pair at 1, 5 and 12 min are shown. Dual whole cell patch clamp methods as described in (Valiunas *et al.* 2002) were employed to allow simultaneous measurement of junctional conductance and the transfer of the fluorescently tagged 12-mer oligonucleotide. The inset in the right-hand panel of A shows the voltage step delivered to the left-hand cell; the step was ± 10 mV (V_1). The right-hand cell was held at 0 mV (V_2) and the junctional current (I_2) was recorded, respectively. The junctional conductance was 40 nS. Inset: vertical bar = 2 nA, horizontal bar = 200 ms. B, lack of transfer of 12-mer in a mouse embryonic fibroblast cell pair imaged after 20 min. Junctional conductance in this example was 30 nS. C, fluorescent image of human mesenchymal stem cells (hMSCs) where one cell has had 12-mer delivered via whole cell patch mode. The junctional conductance was 17 nS. The 12-mer is transferred to two adjacent hMSCs. The 12-mer used in these illustrations (as with all our oligonucleotides) was run on an HPLC to remove any untagged fluorescent probe before experimentation. D, 24-mer oligonucleotide (morpholino) transfer in HeLa Cx43 cell pairs. Fluorescent images were taken at 40 min after the injection of 24-mer into the left-hand cell. To visualize the 24-mer in the recipient cell (right-hand cell) the injected cell was removed. E, both light and fluorescent images of the recipient cell. The measured junctional conductance was 35 nS. Scale bar = 10 μ m.

but not Cx26 or Cx32 gap junction channels, and they suggest the hypothesis that siRNA might behave similarly.

A summary of the data collected from Cx43-expressing HeLa cell pairs using synthetic oligonucleotides is shown in Fig. 2. Figure 2A shows cell-to-cell transfer of the 12-, 16- and 24-mer. The transfer of the double-stranded 12-mer is shown as ▼. As the length of the single-stranded oligonucleotide increased, the permeability decreased. The hybridized 12-mer had significantly lower permeability than the single strand, indicating that the width of the molecule also influences the rate of transfer from cell to cell. This finding is expected for molecules whose minor diameter is close to the pore diameter (Goldberg *et al.* 1999, 2004; Valiunas *et al.* 2002).

In a limited number of experiments, transfer of oligonucleotides and measurement of junctional conductance were performed simultaneously. The data are summarized in Fig. 2B. The X-axis is the junctional conductance and represents increasing numbers of channels while the Y-axis is the fluorescent intensity of the recipient cell relative to the source cell, at a fixed time interval (12 min for 12- and 16-mer, 40 min for the 24-mer). The transfer of nucleotide increased in proportion to junctional conductance, supporting the hypothesis that oligonucleotide transfer in these experiments occurs via gap junctions composed of Cx43.

Cell-to-cell transfer of siRNA

To test whether siRNA would transit gap junction channels, we used three cell lines: (1) NRK cells that endo-

genously express Cx43 (Musil & Goodenough, 1991; Hand *et al.* 2002); (2) mouse embryonic fibroblasts, Mβ16tsA cells, which express Cx32 and Cx26 but no Cx43 (see Fig. 3A); and (3) connexin-deficient N2A cells (Veenstra *et al.* 1995). We do not provide a western blot demonstrating Cx43 expression in NRK cells because their endogenous expression of this connexin has been reported previously (Musil & Goodenough, 1991; Hand *et al.* 2002). For NRK and Mβ16tsA cells the mean \pm s.d. junctional conductance was 18.2 ± 10 ($n = 7$) and 18.1 ± 9 nS ($n = 16$), respectively (N2A parental cells are communication deficient and so no measurement of junctional conductance was made; Veenstra *et al.* 1994).

Dual whole cell patch clamp illustrates gap junction-mediated coupling in Mβ16tsA and NRK cells (Fig. 3B). The data shown in Fig. 3B represent examples of junctional conductances, 4 and 10 nS, respectively, which demonstrate voltage dependence. N2A cells are communication deficient and we show no records as a consequence (Brink *et al.* 1997). We generated a mouse embryonic fibroblast cell line that stably expressed siRNA against pol β (Mβ16tsA- β kcdc). Polosina *et al.* (2004) have reported a Mβ16tsA knockdown in which pol β is 5–10% of normal levels. We also generated an NRK cell line that stably expressed an siRNA that targets pol β . The western blot for NRK-kcdc has $\sim 30\%$ of wild-type protein levels of pol β , as shown in Fig. 4A. The NRK-kcdc cell line also showed an increased sensitivity to the DNA methylating agent methylmethane sulphonate (MMS, Fig. 4B), the hallmark of a pol β deficiency (Sobol *et al.* 1996). Lastly, NRK-kcdc cells were stained with a

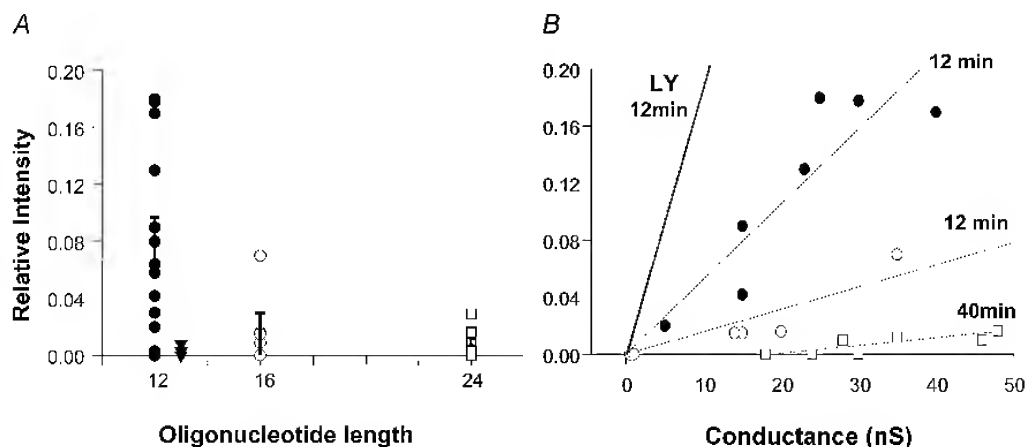


Figure 2. Oligonucleotide permeation declines with increasing length and diameter

A, summary of all transfer data for 12-mer (●), 16-mer (○), 24-mer (□) and 12-mer double-stranded oligonucleotides (▼). The hybridized 12-mer is shifted to the 13-mer position to allow visibility of the data points. As the probes become longer or wider their permeability declines. B, plot of junctional conductance against fluorescence for the subset of experiments in which junctional conductance and oligonucleotide transfer were measured simultaneously (Valiunas *et al.* 2002). All fluorescence measurements were taken at 12 min after dye injection with the exception of the 24-mers (□) which were taken at 40 min.

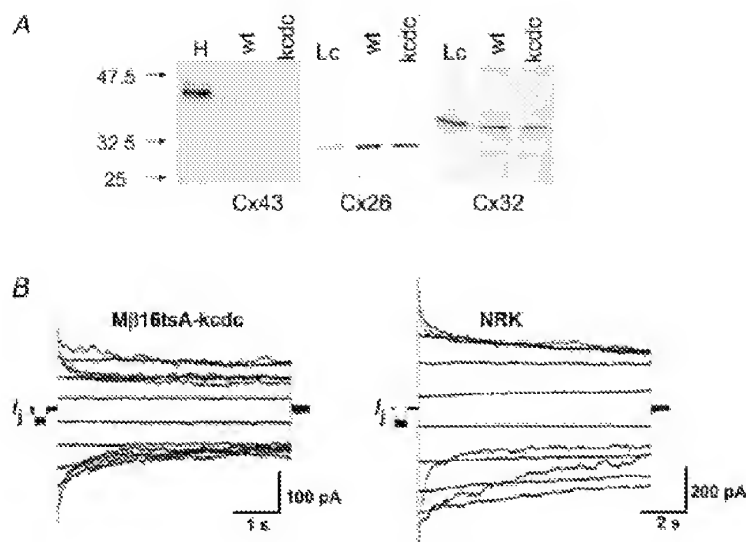


Figure 3. Gap junction-mediated coupling for embryonic mouse fibroblasts (Mβ16tsA-kcdc cells) and NRK-wt cells

A, western blots of Mβ16tsA cells showed a lack of Cx43 but did reveal the presence of Cx32 and Cx26. NRK cells have been shown to express Cx43 endogenously (Hand *et al.* 2002). Left-hand panel, from left to right: H, heart, which is known to express high levels of Cx43; wt, Mβ16tsA-wt cells; and kcdc, Mβ16tsA-kcdc cells. Middle panel, from left to right: Lc, lacrimal gland, which is known to express high levels of Cx26 (Walcott *et al.* 2002); wt, Mβ16tsA-wt cells; and kcdc, Mβ16tsA-kcdc cells. Right-hand panel: Lc, lacrimal gland, which is known to express high levels of Cx32 as well (Walcott *et al.* 2002); wt, Mβ16tsA-wt cells; and kcdc, Mβ16tsA-kcdc cells. B, dual whole cell patch clamp of embryonic mouse fibroblasts or NRK cell pairs revealed coupling for both cell types. Junctional conductance derived from junctional current (I_j) for the embryonic mouse fibroblast shown was 4 nS and for the NRK it was 10 nS. These values represent the low end of the range of measured conductances. They are used to illustrate the voltage-dependent behaviour typical of gap junctions. When junctional conductance is higher the ability to demonstrate voltage dependence is compromised owing to series resistance of the patch electrode.

vital dye (see Methods) and co-cultured with NRK-wt cells for 6–8 days. Mβ16tsA-kcdc cells were stained and co-cultured with Mβ16tsA-wt, N2A cells or NRK-wt cells for 6–8 days. Prior to co-culture, all wt cells expressed normal pol β levels. After co-culturing the mixed populations were separated via fluorescence-activated cell sorting (FACS). Pol β mRNA levels were measured by semiquantitative RT-PCR. Figure 5 shows that pol β expression was reduced in the NRK-wt cells that were co-cultured with NRK-kcdc cells, but was unaffected in the Mβ16tsA-wt, N2A and NRK-wt

cells after co-culture with Mβ16tsA-kcdc cells. Figure 5A shows RT-PCR results for co-culturing of NRK-wt and NRK-kcdc cells (left-hand panel) where FACS demonstrates knockdown in the wt cells. Co-cultured Mβ16tsA-wt and Mβ16tsA-kcdc cells (right-hand panel) failed to show a knockdown in the wt cells after FACS. This result demonstrates that cells which are electrically coupled with Cx32 and Cx26 are incapable of effective transfer of siRNA. Figure 5B shows the lack of knockdown, from left to right, for N2A cells co-cultured with Mβ16tsA-kcdc cells, NRK-wt cells co-cultured with Mβ16tsA-kcdc cells

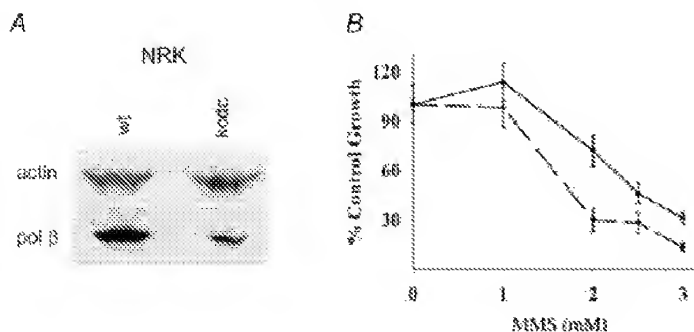


Figure 4. Knockdown of DNA polymerase β (pol β) in NRK cells

A, NRK cells stably transfected with pHPkneoB (NRK-kcdc) have a 70% decrease in pol β protein levels by immunoblotting when compared to untransfected NRK-wt cells. B, NRK-kcdc cells are more sensitive to the DNA-damaging agent, MMS. Dashed line represents cells transfected with siRNA for pol β.

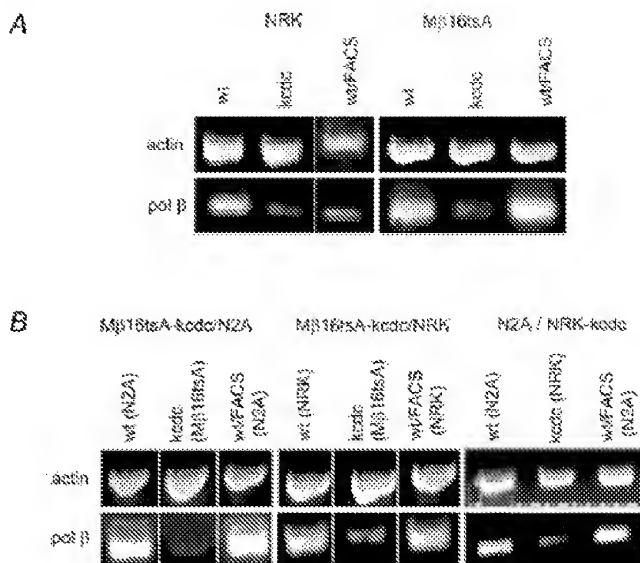


Figure 5. siRNA was transferred into NRK-wt cells expressing Cx43 but not into Mβ16tsA cells expressing Cx26 and 32

A, from left to right: NRK-wt and NRK-kcdc cells; or Mβ16tsA-wt and Mβ16tsA-kcdc cells. wt/FACS indicates NRK-wt cells or Mβ16tsA-wt cells after separation via FACS. B, from left to right: N2A and Mβ16tsA-kcdc; NRK-wt and Mβ16tsA-kcdc; and N2A and NRK-kcdc. wt/FACS indicates N2A or NRK-wt cells after separation via FACS. In all cases the cells were co-cultured for 6–8 days and subsequently fluorescent wt cells were isolated by FACS, and pol β mRNA levels of the wt cells as well as the kcdc cells were determined by RT-PCR. Only NRK cells exhibit transfer of siRNA, as shown by the decrease in the NRK-wt pol β mRNA levels (in A).

or N2A cells co-cultured with NRK-kcdc cells. In this case there should be no electrical coupling (since N2A cells are connexin deficient) and there is no siRNA transfer. The data presented in Fig. 5B eliminate the possibility that an extracellular pathway mediates the transfer of siRNA based on the expectation of little or no cell-to-cell coupling mediated by gap junction channels in these cases (Brink *et al.* 1997; Gemel *et al.* 2004). One significant feature associated with successful siRNA transfer to NRK-wt cells from NRK-kcdc cells is the presence of Cx43. This connexin was absent in Mβ16tsA-wt and Mβ16tsA-kcdc mouse fibroblasts expressing Cx32 and Cx26. This suggests that siRNA can permeate through gap junction channels composed of Cx43 but not those composed of Cx32 or Cx26.

We obtained further evidence illustrating delivery of siRNA to NRK-wt cells in experiments in which the ratio of wild type to kcdc cell was varied. Figure 6 is a bar graph summarizing data from five experiments in each condition using NRK-wt and NRK-kcdc cells at a 1:1 and 1:2 ratio. The bar graph is calculated as percentage knockdown where NRK-wt is 0% and NRK-kcdc is 100%. Co-culturing at 1:1 resulted in a reduction in pol β of $25 \pm 17\%$ ($P < 0.2$, not significant). The 1:2 ratio resulted in almost complete knockdown ($96 \pm 4\%$ ($P < 0.001$)). These results suggest that the degree of knockdown will depend on the ratio of wild type to knockdown cells.

Discussion

Previously it was thought that solutes of approximately 1.0–1.5 kDa represented the upper limit for permeation through gap junction channels (Simpson *et al.* 1977; Loewenstein, 1981; Neijssen *et al.* 2005). Our studies show that synthetic oligonucleotides, in the form of

morpholinos with molecular weights of ~2–4 kDa, minor diameters of 1.0–1.1 nm and lengths of 7.6 nm, are able to diffuse from cell to cell via Cx43 channels. As far back as 1971 Kolodny suggested that 3T3 cells could transfer mRNA via gap junctions (Kolodny, 1971). Our data strongly suggest that even though these cells make Cx43 (Carystinos *et al.* 2003), his result was rather a demonstration of transfer of single nucleotides or possibly small oligonucleotides. In addition, siRNA for pol β can be effectively transferred. In all cases the likely forms would be non-hybridized and to a lesser extent hybridized forms since they are less permeant. It is unlikely that hairpin forms of siRNA are transferred based on the effective action of Dicer enzymes (Shankar *et al.* 2005), which cleave hairpin siRNA leaving only non-hybridized and hybridized forms. In addition, the shape of the hairpin

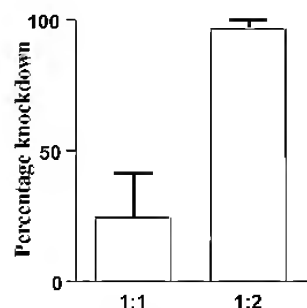


Figure 6. siRNA delivery depends on the ratio of wild type to knockdown cells

RT-PCR for pol β in NRK-wt cells co-cultured with NRK-kcdc cells at ratios of 1:1 or 1:2 in five separate experiments at each ratio. The co-cultured cells were separated via FACS. The bar graph depicts the percentage knockdown for the 1:1 ratio and the 1:2 ratio. At a ratio of 1:2 the knockdown is almost complete, leaving pol β levels equal to those in NRK-kcdc cells. Standard error bars are shown.

siRNA should limit permeation. Quantitative analysis of the co-culture of NRK-kcdc (knockdown cells) with NRK-wt shows a reduction in pol β expression in the NRK-wt for the 1:1 ratio (wild-type to kcdc) which was not statistically significant but was highly significant when the ratio was one NRK-wt to two NRK-kcdc (1:2).

The potential for transfer of oligonucleotides via the extracellular space seems unlikely because Cx43 was the only connexin that was permeable to morpholinos and siRNA for pol β . This is unlike other connexin-induced extracellular pathways, where Cx43, Cx26 and Cx32 all trigger extracellular-mediated cell-to-cell communication via the opening of hemichannels (Tran Van Nhieu *et al.* 2003). Another unlikely possibility requires that the Cx43-expressing cells have higher rates of pinocytosis relative to cells expressing other connexins.

For the morpholinos, degradation resulting in the release of the tag on the 3' end seems unlikely based on previous studies (Summerton & Weller, 1997), and if there were cytoplasmic-dependent breakdown into the cytoplasm then the flux from source cell to recipient cell would be expected to be independent of morpholino length. This was not the case.

Obviously, this is not an all-inclusive study and there may be other connexins that allow passage of siRNA. The implication is that, through regulation of connexin expression, a tissue can regulate its pattern of gene expression. This potential role for gap junctions has not been appreciated previously. In principle, a small group of cells or possibly a single cell within a syncytium has the potential to affect gene expression in a larger group of cells or possibly an entire organ. Moreover, this phenomenon suggests the possibility of introducing cells expressing specific siRNAs to therapeutically modify gene expression in a target organ or a tumour. The data presented here suggest that siRNA against pol β will transfer from a pol β knockdown cell to a wild type cell through gap junction channels composed of Cx43 but not Cx32/Cx26. The length (22-mer) of siRNA against pol β is typical of most other siRNAs, so if it can move from cell to cell, other RNA molecules should do so as well. To test this hypothesis, fluorescently labelled oligonucleotides, simulating siRNAs, were generated (12-mer, 16-mer and 24-mer). They too were found to permeate gap junction channels composed of Cx43 but not Cx26/Cx32. These data support the novel hypothesis that gap junction channels have connexin-specific permeability to siRNA.

These experiments also suggest that cellular delivery of siRNA might have potential as a therapeutic tool (Lieberman *et al.* 2003; Berkhout, 2004). The major barrier to clinical application has been a means for *in vivo* delivery of exogenous siRNA to the interior of the target cell (Zhang *et al.* 2004). We have recently shown that human mesenchymal stem cells make connexins (including Cx43)

and form gap junctions *in vitro* and *in vivo* (Valiunas *et al.* 2004; Potapova *et al.* 2004), raising the possibility of their use as an autologous cell-based delivery system for gene suppression. Data presented here suggest that Cx43-expressing mesenchymal stem cells will transfer siRNA to other Cx43-expressing cells. At this stage, we have identified only Cx43 channels as being permeable to siRNA. Cx43 is ubiquitously expressed in vertebrates. More extensive studies may identify other connexins that form siRNA-permeable channels. Another potential limitation is the rate of degradation of the siRNA. The extent to which siRNA can diffuse through a syncytium depends on both the rate of cell-to-cell transfer through gap junctions and the rate of intracellular degradation. Because of its relatively large size, siRNA diffuses slowly through gap junctions formed from Cx43, thus the rate of degradation would have to be slow to allow extensive diffusion. However, in invertebrates, siRNA-recipient cells have the ability to self-replicate the siRNA (Fire *et al.* 1998; Lipardi *et al.* 2001). If a similar mechanism exists in mammals, then siRNA-producing cells introduced into a syncytium could trigger an effective propagation of the siRNA throughout that tissue. Many questions remain, but our data clearly show that siRNA can be delivered from the interior of one cell to that of another independent of the extracellular space and influence gene expression in the recipient cell.

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